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Amino acid substitutions in the *Candida albicans* sterol $\Delta^{5,6}$-desaturase (Erg3p) confer azole resistance: characterization of two novel mutants with impaired virulence

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Objectives: To determine the mechanisms responsible for fluconazole resistance in two *Candida albicans* isolates (CAAL2 and CAAL76) recovered from two hospitalized patients after fluconazole prophylaxis.

Methods: MICs of fluconazole and voriconazole were determined by the broth microdilution method (CLSI M27-A3), and by Etest® for amphotericin B. RNA expression levels of *CDR1*, *MDR1* and *ERG11* were determined by RT–PCR. Mutations in *ERG11* and *ERG3* were investigated by amplification and sequencing. Sterol membrane profiles were determined by gas chromatography–mass spectrometry (GC–MS). *In vivo* virulence was determined in a murine model of invasive candidiasis.

Results: Both isolates displayed azole cross-resistance and reduced susceptibility to amphotericin B, and are novel $\Delta^{5,6}$-desaturase (Erg3p) mutants. CAAL2 harbours a new amino acid substitution (L193R), whereas a 13 bp deletion leading to a truncated Erg3p (Δ366–378) was found in CAAL76. Both genetic alterations impaired Erg3p function as shown by GC–MS in these isolates (ergosterol content below 10%, and accumulation of ergosta-7,22-dienol above 40%). *In vivo*, in a murine model of invasive candidiasis, both CAAL2 and CAAL76 exhibited a significant trend toward reduced virulence, which seems to be linked to a reduced capacity for hyphal growth.

Conclusions: These findings demonstrate the critical role of residue 193 in Erg3p function and azole resistance. We suggest that this attenuated *in vivo* virulence phenotype could be linked to lower potential for hyphal growth. Taken together, our findings highlight the fact that *erg3* mutants must be considered in future studies aiming at investigating azole antifungal drug resistance.

Keywords: *C. albicans*, azole antifungals, amphotericin B, *ERG3*, ergosterol biosynthesis

Introduction

Candida species are major human pathogens responsible for a wide spectrum of diseases ranging from superficial to life-threatening invasive infections. Whereas more than 40 species have been described as human pathogens, *Candida albicans* still remains the main species responsible for candidaemia worldwide.[1,2] Azole antifungal agents represent, with the echinocandins, the most widely used treatments of candidiasis. However, acquisition of resistance can occur, leading to the risk of breakthrough infections.[3,4] In recent years, intense research has been conducted, leading to a better understanding of the molecular mechanisms of azole resistance, among which the most prevalent in *C. albicans* are: (i) reducing intracellular concentration of the azole drugs by overexpression of efflux pumps belonging to either the ATP-binding cassette superfamily (*CDR1*, *CDR2*) or the major facilitator superfamily (*MDR1*); (ii) up-regulation of the *ERG11* gene encoding 14α-lanosterol demethylase, the primary target of azole drugs; and (iii) point mutations in *ERG11* causing amino acid changes that lead to decreased affinity for azole drugs.[5] Importantly, these mechanisms are frequently combined in a single isolate.[6–8] An important step toward a better understanding of genetic regulation of azole resistance in *C. albicans* has been recently achieved with the discovery of hyperactive alleles (resulting from gain-of-function mutations) in the zinc-finger transcription factor genes encoding *TAC1*, *MRR1* and *UPC2*, which are responsible for the constitutive overexpression of *CDR1*/*CDR2*, *MDR1* and *ERG11*,

respectively.[9–11] However, whereas active efflux and amino acid substitutions in Erg11p are the most common mechanisms of azole resistance in *C. albicans*, other poorly studied mechanisms involving other enzymes of the ergosterol biosynthesis pathway, such as Erg3p and Erg5p, can occur.[12–16]

In a previous study we investigated *ERG11* polymorphism in a collection of *C. albicans* clinical isolates, allowing us to describe novel mutations that were recently implicated in azole resistance, by site-directed mutagenesis.[17,18] Interestingly, two azole-resistant clinical isolates from this collection (CAAL2 and CAAL76) exhibited Erg11p substitutions that were previously described in azole-susceptible isolates (D116E, K128T and D153E), suggesting that these mutations could not explain their azole resistance. Here, we show that both *C. albicans* isolates are in fact novel *erg3* mutants that display defective sterol $\Delta^{5,6}$-desaturase activity and reduced susceptibility to amphotericin B. Moreover, our data underline that in addition to their implication in azole resistance, the new *ERG3* mutations impact on *in vivo* virulence. Finally, our data underline that genetic alterations in *ERG3* could arise following fluconazole therapy.

Materials and methods

Strains

CAAL2 and CAAL76 clinical isolates were recovered from two hospitalized patients.[18] Both patients had received fluconazole as antifungal prophylaxis before isolation of the strains. CAAL2 was isolated from the respiratory tract of a 63-year-old heart transplant recipient, while CAAL76 was isolated from a blood culture of a 27-year-old hematopoietic stem cell transplant recipient. Both strains were stored at −80°C for long-term storage.

A set of 10 fluconazole-susceptible clinical isolates from a previous study was used to determine the background expression levels of *PMA1*, *ACT1*, *MDR1*, *CDR1* and *ERG11*.[18] Three of them (CAAL94, CAAL96 and CAAL97) were also used as controls for gas chromatography–mass spectrometry (GC–MS), phenotypic tests and *in vivo* experiments.

Antifungal susceptibility testing

MICs of fluconazole and voriconazole were determined for each isolate using the broth microdilution method as recommended by the CLSI document M27-A3.[19] Fluconazole was obtained from Sigma–Aldrich (Saint-Quentin Fallavier, France), and voriconazole was obtained from Pfizer. MICs were determined visually after 48 h of incubation at 35°C. MIC was considered as the lowest drug concentration that resulted in 50% growth inhibition relative to the growth in the control well. MIC values were compared with the recently proposed revised species-specific clinical breakpoints for fluconazole and voriconazole.[20,21] Briefly, for fluconazole, MICs ≤2 mg/L were considered susceptible (S), 4 mg/L was susceptible dose-dependent (SDD) and ≥8 mg/L was resistant (R); for voriconazole, MICs ≤0.125 mg/L were considered S, 0.25–0.5 mg/L was SDD, and ≥1 mg/L was R. *In vitro* susceptibility to amphotericin B was determined by Etest® (bioMérieux, Marcy l'Étoile, France), this method having shown better performance over microdilution methods for the detection of amphotericin B resistance.[22] The MIC of amphotericin B was defined as the lowest concentration at which fungal growth was completely inhibited, as recommended by the manufacturer.

DNA extraction and ERG3 sequencing analysis

DNA extraction was conducted using a NucleoSpin Tissue kit (Macherey–Nagel, Germany), after cell lysis using lyticase (Sigma–Aldrich). DNA extracts were stored at −20°C until analysis. All primers used in this study are listed in Table S1 (available as Supplementary data at *JAC* Online). PCR amplification of the entire coding sequence of *ERG3* (1161 bp) was obtained using the primers CaERG3F and CaERG3R.[15] All PCR products were purified, and sequencing was performed using a BigDye terminator sequencing kit on an ABI Prism® 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequences were analysed using SeqScape software (Applied Biosystems) and compared with a wild-type *ERG3* reference sequence (GenBank accession numbers AF069752).

Membrane sterol analysis

Analysis of membrane sterol composition was conducted by GC–MS as previously described.[23] Briefly, cells were incubated in 50 mL of Sabouraud dextrose broth (Sigma–Aldrich) at 35°C with stirring for 18 h. Cells were collected by centrifugation at 1500 **g**. The pellet was suspended in 3 mL of saponification medium (25 g of KOH, 36 mL of distilled H_2O and made up to 100 mL with 100% EtOH). Then, the suspension was vortexed for 1 min and incubated at 80°C for 60 min. Sterols were then extracted by addition of a mixture of 1 mL of distilled H_2O and 4 mL of *n*-hexane (Merck, Darmstadt, Germany). Hexane extract was then evaporated. Samples were derivatized with 100 μL of silylating mixture (Fluka, Saint Quentin Fallavier, France) at room temperature (RT) for 30 min, evaporated, and diluted in 500 μL of *n*-hexane. A 2 μL aliquot was injected into a gas chromatograph (model 6890N, Agilent Technologies, Palo Alto, CA, USA) coupled to a quadrupole mass spectrometer (model 5973i, Agilent Technologies). Sterols of interest were identified by their mass spectra. To compare the sterol profiles of the isolates, the AUC of each sterol peak was used to calculate the ratio sterol AUC/sum of sterol AUCs. Three fluconazole-susceptible *C. albicans* isolates (CAAL94, CAAL96 and CAAL97) were included in the experiment as controls.

RNA extraction and RT–PCR transcript levels

RNA was extracted from mid-log phase growth cultures in YPD medium (2% bactopeptone, 1% yeast extract, 1% glucose) at 30°C and 200 rpm, using a NucleoSpin RNA II kit (Macherey–Nagel, Hoerdt, France) after cell lysis using lyticase (Sigma-Aldrich). RNA extracts were aliquotted and stored at −80°C before analysis. Absence of DNA contamination was checked, and RNA integrity was verified using a Bioanalyzer 2100 (Agilent Technologies) with an RNA Nano 6000 kit (Agilent). RNA expression levels of *ERG11*, *MDR1* and *CDR1* as well as of two housekeeping genes (*PMA1* and *ACT1*) were determined by quantitative RT–PCR on a Rotor-Gene 3000 instrument (Corbett Life Science, Sydney, Australia) using a One Step PrimeScript RT–PCR kit (Takara Bio Inc., Saint-Germain-en-Laye, France) with primers and probes described in Table S1. Relative expression was measured quantitatively after normalization to an 18S rRNA control (Applied Biosystems) by simultaneous amplification with each target gene as described previously.[7] Each RNA sample was tested in triplicate. RNA transcript levels of CAAL2 and CAAL76 were compared with the normal expression range of a collection of 10 fluconazole-susceptible *C. albicans* isolates (Table S2, available as Supplementary data at *JAC* Online). Overexpression was considered significant when ΔCt ($Ct_{gene\ of\ interest} - Ct_{18S\ rRNA}$) exceeded 3 SD.

Murine model of invasive candidiasis

Four-week-old female Swiss mice (Centre d'élevages Janvier, Le Genest St Isle, France) were immunocompromised by subcutaneous administration of 30 mg/kg prednisolone (Sigma–Aldrich) one day before infection. Disseminated candidiasis was induced by inoculation with 5×10^6 *C. albicans* cells in 0.1 mL yeast suspension into the lateral tail vein. Inoculum size was determined in preliminary studies for the fluconazole-susceptible

strains with the aim to obtain lethality by between 4 and 7 days. The same conditions were used for inoculation with *erg3* mutants. Survival was monitored every day for 10 days after infection. Three fluconazole-susceptible isolates were used as control (CAAL94, CAAL96 and CAAL97). The survival of animals infected with each *erg3* mutant isolate was compared with those of animals infected with each fluconazole-susceptible isolate using the log rank test. In addition, groups of animals infected with wild-type isolates and those with *erg3* mutant isolates were compared with the same statistics test. A P value <0.05 was considered significant.

This study was carried out in strict accordance with the recommendations of the Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes. The protocol was approved by the Committee on Ethics of Animal Experiments of the Experimental Therapy Unit (UTE) of the Faculty of Medicine, University of Nantes, France (C-44015).

Germ tube formation and hyphal growth in vitro

The capacity of the isolates to produce germ tubes was investigated after incubation of the isolates at 35°C in human serum for 2 h without shaking. Production of pseudohyphae was analysed by subculturing the isolates on Potato Carrot Bile Agar medium (PCB) at 30°C for 24–48 h. The kinetics of hyphal growth was investigated as follows. For each isolate, 100 μL of a suspension (with a turbidity equivalent to that of a 3 McFarland standard) in distilled water was incubated for 16 h at 35°C with 1 mL of RPMI 1640 liquid medium (Sigma–Aldrich) supplemented with 10% fetal bovine serum (FBS; Sigma–Aldrich). Three fluconazole-susceptible isolates (CAAL94, CAAL96 and CAAL97) were included in each experiment as controls.

Nucleotide sequence accession numbers

ERG3 nucleotide sequences of the two isolates have been deposited in the GenBank database under accession numbers JN565145 and JN565146.

Results

As shown in Table 1, both *C. albicans* isolates displayed high levels of resistance to fluconazole (>64 mg/L) and voriconazole (>8 mg/L), with MICs largely above the recently revised clinical susceptibility breakpoints. Both strains harbour mutations leading to amino acid substitutions in Erg11p (D116E, K128T and D153E) that have been previously described in azole-susceptible isolates, suggesting that these mutations cannot account for azole resistance in our isolates. Strikingly, CAAL2 and CAAL76 displayed an azole-resistant phenotype that seemed to depend neither on *CDR1*- nor on *MDR1*-mediated efflux, as revealed by measurements of mRNA transcripts by RT–PCR (Table 2). However, both isolates displayed significant *ERG11* overexpression (2.9- and 4.2-fold, respectively, relative to the set of fluconazole-susceptible isolates).

Besides fluconazole and voriconazole resistance, CAAL2 and CAAL76 also displayed unusually high MICs of amphotericin B (0.75 and 1 mg/L, respectively), a phenotype that has been previously linked to a defect in Erg3p.[16] Therefore, to gain further insight into the molecular mechanisms responsible for azole resistance in these isolates we amplified the *ERG3* coding sequence. Each isolate displayed a novel genetic alteration in *ERG3*. In addition to the V351A amino acid change that has been previously reported from azole-susceptible isolates, CAAL2 displayed a novel homozygous T578G nucleotide substitution, leading to a leucine-to-arginine exchange (L193R) in Erg3p (Table 1). Interestingly, L193R occurred at a residue that is highly conserved in yeasts.[24] CAAL76 displayed a large, homozygous, 13 bp deletion, from nucleotides 366 to 378, responsible for a frameshift and truncated protein due a premature stop codon.

To investigate whether the observed Erg3p amino acid substitutions could impact on ergosterol biosynthesis, we determined the membrane sterol profiles of these isolates by GC–MS. As shown in Table 3, each of the three fluconazole-susceptible *C. albicans* isolates (CAAL94, CAAL96 and CAAL97) displayed a normal sterol profile, with ergosterol content representing more than 90% of the total sterol fraction, and a low amount of sterol intermediates. In stark contrast, the two azole-resistant strains displayed very low ergosterol content: <10% for CAAL2 and not detectable for CAAL76. Interestingly, both strains displayed accumulation of ergosta-7,22-dienol (64.2% and 41% for CAAL2 and CAAL76, respectively) and other sterol intermediates such as episterol (10.4% and 33.5%, respectively) and ergosta-7-enol (8% and 4.3%, respectively), highlighting a defective sterol $\Delta^{5,6}$-desaturase activity in these isolates.

We therefore investigated the virulence of these isolates in a murine model of invasive candidiasis to study whether the genetic alterations found in *ERG3* could have an impact on *in vivo* virulence. As depicted in Figure 1, despite slight differences in survival between wild-type isolates, a significant trend toward a reduced virulence of both *erg3* mutants was noted by comparison with wild-type isolates ($P<0.005$). Further investigations revealed that both *erg3* mutants exhibited the typical yeast form on Sabouraud dextrose agar, and retained the ability to form germ tubes, pseudohyphae and hyphae. However, kinetics studies revealed, under our conditions, a clearly reduced capacity

Table 1. Results of antifungal susceptibility testing and mutations leading to amino acid substitutions in Erg11p and Erg3p for the two *C. albicans* clinical isolates

		MIC (mg/L)				
	Site of isolation	FLC	VRC	AMB	Mutations in Erg11p	Mutations in Erg3p
CAAL2	respiratory tract	>64	>8	0.75	D116E[a], D153E[a]	L193R, V351A
CAAL76	blood	>64	>8	1	D116E[a], K128T[a]	ΔL122-I125[b]

FLC, fluconazole; VRC, voriconazole; AMB, amphotericin B;
[a]Heterozygous; other mutations are homozygous.
[b]Deletion from nucleotides 366 to 378 in *ERG3* resulted in a frameshift and truncated Erg3p due to a premature stop codon.

Table 2. RNA transcript levels of *PMA1*, *ACT1*, *ERG11*, *MDR1* and *CDR1* of the two *C. albicans* clinical isolates, as determined by RT–PCR

	ΔCt				
	PMA1	*ACT1*	*ERG11*	*MDR1*	*CDR1*
CAAL2	9.91	11.02	**11.11 (×2.9)**	12.99	14.19
CAAL76	10.15	11.20	**10.57 (×4.2)**	12.86	13.28

Values that fall outside the 3 SD range are shown in bold.

Table 3. Membrane lipid sterol composition of *erg3* mutant isolates, as determined by GC–MS

	Percentage of each sterol fraction in the *C. albicans* isolates				
	CAAL2	CAAL76	CAAL94	CAAL96	CAAL97
Lanosterol/obtusifoliol[a]	1.2	1.5	—	—	—
Eburicol	2.2	—	—	—	—
Zymosterol	—	1.2	1.3	2.0	1.2
Fecosterol	6.2	18.2	—	—	—
Episterol	10.4	33.5	—	—	1.0
Ergosta-7,22-dienol	**64.2**	**41.0**	—	—	—
Ergosta-7-enol	8.0	4.3	—	—	—
Ergosta-5,8-dienol	—	—	5.0	—	—
Ergosterol	6.0	—	**90.8**	**94.7**	**95.9**
Unidentified	1	—	—	—	1.8

A sterol amount below 1% (or not detected) is represented by —.
All three fluconazole-susceptible isolates (controls) have the *ERG3* wild-type sequence.
The most abundant sterol for each isolate is shown in bold.
Data are the average of three independent experiments only for CAAL2.
[a]Lanosterol and obtusifoliol cannot be differentiated (similar molecular weights).

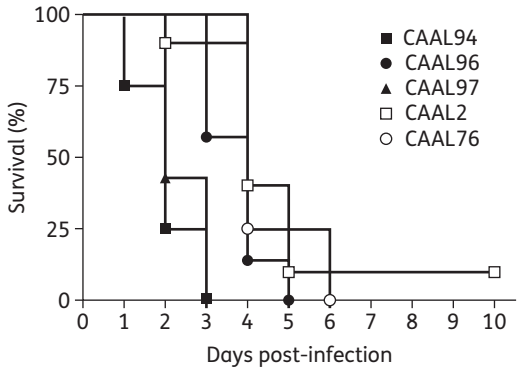
for hyphal growth of these mutants in comparison with the wild-type isolates (Figure 2).

Discussion

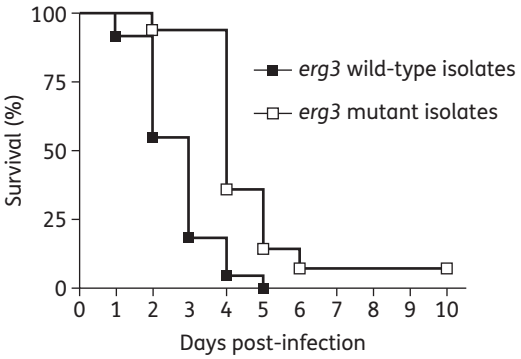
Acquisition of azole resistance, described in several species, is of serious concern in *Candida*, leading to treatment failures, and even to the latest generation of drugs, such as voriconazole and posaconazole.[3,4] Up to now, a large number of studies have focused on the mechanisms leading to azole resistance in *C. albicans*, especially up-regulation of the genes encoding efflux pumps and amino acid substitutions in Erg11p.[25] So far, the impact of genes, apart from *ERG11*, involved in the ergosterol biosynthesis pathway in azole resistance (such as *ERG3* and *ERG5*) has been poorly investigated.[14,15] In the present study, we report two novel *C. albicans* isolates with an *erg3* mutant phenotype, isolated from two patients receiving fluconazole prophylaxis.

Sterol $\Delta^{5,6}$-desaturase, encoded by *ERG3*, is a key enzyme in the ergosterol biosynthesis pathway, being responsible for introducing a double bond in the ring structure of episterol to produce ergosta-5,7,24(28)-trienol. The latter, an intermediate in ergosterol biosynthesis, is the substrate of sterol Δ^{22}-desaturase and sterol Δ^{24}-reductase (encoded by *ERG5* and *ERG4*, respectively).[26] Initially studied in *Saccharomyces cerevisiae*, *erg3* null mutants are intrinsically resistant to azole drugs.[27] In *Candida* species, mutations in *ERG3* protect yeast cells from membrane damage due to the accumulation of 14α-methylfecosterol instead of the toxic product 14α-methylergosta-8,24-dien-3β,6α-diol that results from *ERG11* inhibition by azole drugs. In most of the studies published to date, *erg3* mutant isolates displayed cross-resistance between azoles and polyenes, such as amphotericin B, both of which are drug classes that target ergosterol.[13,15,16,26,28] However, according to a recent study, the reduced susceptibility to amphotericin B could be infrequent: mutants called 'leaky' are low-ergosterol-producing isolates due to a slight *ERG3* defect.[15] Here, CAAL2 and CAAL76 could be considered as typical *erg3* mutants, exhibiting extremely low ergosterol content (not detectable for CAAL76) and resistance to azoles and amphotericin B.

Apart from those of *C. albicans* and its related species *Candida dubliniensis*, *erg3* mutants have been described in a limited number of *Candida* species.[29–31] Moreover, only a few of these *erg3* mutants have been investigated for mutations leading to amino acid changes in *ERG3*.[7,15,28,30,32,33] Up to now, 16 genetic alterations (mostly amino acid substitutions) have been reported in *C. albicans* isolates (Table 4). Apart from D19E, obtained *in vitro* by serial cultures of a wild-type strain in the presence of fluconazole, all other mutations have been reported from clinical isolates.[33] Importantly, with a few exceptions (e.g. W332R), most of these substitutions have been described in combined mutations, in azole-resistant isolates. Thus, their exact involvement as a single substitution in Erg3p impairment and azole resistance remains to be established. Other amino acid changes (Q160K, H269N, P272L, H318N and Q327K) have been reported only in *C. dubliniensis* (Table 4).[30] Here, in addition to V351A, which occur at a residue that is probably not crucial for Erg3p function,[15] we describe two novel genetic alterations associated with impaired $\Delta^{5,6}$-desaturase activity, as witnessed by the very low ergosterol content and significant accumulation of ergosta-7,22-dienol in the corresponding isolates. Interestingly, a substitution at residue 193 (L193P) has been identified recently by Martel and co-workers in a single *C. albicans* isolate exhibiting an *erg3* mutant phenotype.[15] However, in this work, the exact role of L193P in azole resistance could not be determined because of the multiple amino acid changes in this isolate (K97E, V237A, V351A and A353T). Here, we described a different substitution at the same residue (L193R). L193R was homozygous in CAAL2. Taken together, these findings highlight the critical role of residue 193 in Erg3p function and suggest that substitution at this residue can confer azole resistance and reduced susceptibility to amphotericin B through Erg3p impairment. Notably, the sterol membrane pattern of CAAL76, as evidenced by GC–MS, showed a loss of sterol $\Delta^{5,6}$-desaturase activity; this was in complete agreement with *ERG3* gene sequencing, which showed a major deletion leading to a truncated Erg3p. Indeed, loss of Erg3p activity in CAAL76 can be explained by the frameshift deletion, leading to the loss of a large part of the 5′ end of the protein including the four histidine-rich motifs. These domains, which are conserved among all known



P values	CAAL2	CAAL76
CAAL94	<0.001	0.0023
CAAL96	0.1281	0.1521
CAAL97	<0.001	0.0030



P value	*erg3* mutant isolates
erg3 wild-type isolates	<0.001

Figure 1. Survival of mice infected with CAAL2, CAAL76 or wild-type fluconazole-susceptible isolates. For each strain, 5×10^6 blastoconidia (except for CAAL2, 3×10^6) were injected into the tail vein. The following numbers of mice were used: CAAL2, $n=10$; CAAL76, $n=4$; CAAL94, $n=8$; and CAAL96 and CAAL97, $n=7$. Left panel: survival rate of each *erg3* mutant isolate was compared with each fluconazole-susceptible isolate. Right panel: survival rate of *erg3* mutant isolates (grouped together) was compared with wild-type isolates (grouped together). *P* values <0.05 were considered significant (log rank test).

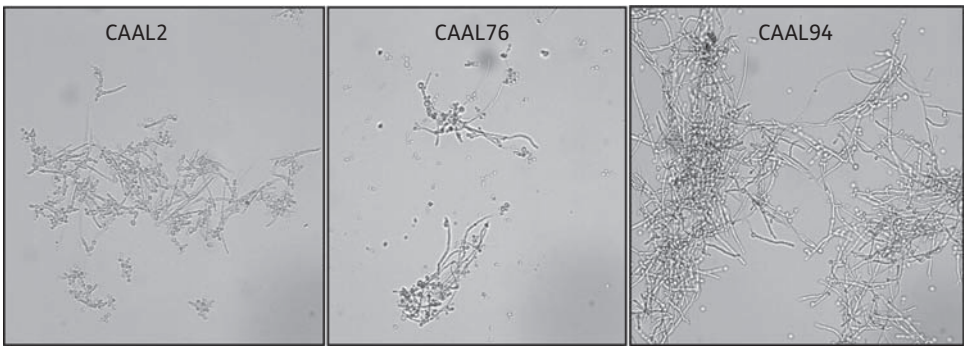


Figure 2. Kinetics of hyphal formation for CAAL2 and CAAL76 in RPMI 1640/10% FBS. Note that under these conditions, both CAAL2 and CAAL76 exhibit a clearly reduced hyphal growth by comparison with the wild-type azole-susceptible isolate (CAAL94) used as a control.

sterol $\Delta^{5,6}$-desaturases, are suspected to comprise the active site of the enzyme.[30,32] Whereas *ERG3* mutations have been previously reported in *C. albicans* in patients following fluconazole therapy, to the best of our knowledge, deletion leading to a frameshift and truncated $\Delta^{5,6}$-desaturase has been mainly observed in *C. dubliniensis* after *in vitro* experiments that aimed at selecting itraconazole-resistant mutants, but only in a single *C. albicans* isolate.[12,28,30,32] Notably, in these strains, deletions were smaller (2 bp) than the deletion observed in CAAL76 (13 bp). Both *erg3* mutant isolates also displayed significant *ERG11* overexpression, confirming that *ERG11* is usually induced, possibly as a compensatory mechanism in *erg3* mutant isolates.[26,29,30,34,35]

As shown in previous studies, yeast lacking functional Erg3p remains viable, as this gene is non-essential for growth in aerobic conditions, but can exhibit reduced growth rate.[26,29,36] Furthermore, studies investigating morphological changes associated with Erg3p loss of function, showed that some *erg3* mutants had lost the capability to produce hyphae.[12,34] Thus, some authors have suggested that this phenotypic characteristic is responsible for the reduced *in vivo* virulence of these mutants.[12,34] While Martel and coworkers showed that some *erg3* mutant isolates can still produce hyphae, their *in vivo* virulence was unfortunately not investigated.[15] In line with this, some controversy regarding the clinical significance of these mutants has arisen.[12,26,34] Recently, Vale-Silva and coworkers have shown, in an immunocompetent BALB/c mouse model, that loss of Erg3p activity does not necessarily decrease virulence.[28] As suggested by the authors, the reduced *in vivo* fitness of these isolates could be compensated for by as yet unknown mechanisms.[28] In the present study, our results clearly confirm that *C. albicans* isolates can retain the ability for hyphal growth despite sterol $\Delta^{5,6}$-desaturase impairment.[15,28] Finally, our experiments, in an immunosuppressed Swiss mice model, also revealed (even in the absence of a complete loss of ability to form hyphae) a significantly lower *in vivo*

Table 4. Erg3p amino acid substitutions previously described in *C. albicans* and *C. dubliniensis*

Erg3p amino acid substitution[a]	Notes	References
C. albicans		
D19E[b,c]	occurred as a single mutation	33
-[c,d]	2 bp deletion (nucleotides 120 to 121) leading to a frameshift and truncated protein (stop codon at position 52)	28
K97E	associated with other substitutions (L193P, V237A and A353T)	15
D147G	associated with T330A (leaky *erg3* mutant); residue highly conserved in yeasts	15
-[c]	13 bp deletion (nucleotides 366 to 378) leading to a frameshift and truncated protein (stop codon at position 153)	this study
A168V[d]	associated with T329S (Darlington strain); residue highly conserved in yeasts	32
L193P	associated with other substitutions (K97E, V237A and A353T); residue highly conserved in yeasts	15
L193R[c]	associated with V351A; residue highly conserved in yeasts	this study
W228Stop[c]	occurred as a single mutation; residue highly conserved in yeasts	7
V237A	associated with other substitutions (K97E, L193P and A353T)	15
H243N	associated with T330A (leaky *erg3* mutant); residue highly conserved in yeasts	15
L266Stop[c]	occurred as a single mutation; residue highly conserved in yeasts	7
W292Stop	associated with other substitutions (Darlington strain)	32
T329S[d]	associated with A168V (Darlington strain); residue highly conserved in yeasts	32
T330A	associated with D147G or H243N (leaky *erg3* mutant); residue highly conserved in yeasts	15
W332R[c]	occurred as a single mutation; residue highly conserved in yeasts	15
V351A	recovered in both azole-susceptible and azole-resistant isolates	this study, 32
A353T	associated with other substitutions (K97E, L193P and V237A)	15
C. dubliniensis		
Q47H[b]	recovered in an azole-susceptible isolate	30
V119I[b]	recovered in an azole-susceptible isolate	30
-[b,c,d]	2 bp deletion leading to a frameshift and truncated protein (stop codon at position 147)	30
Q160K[b,c,d]	residue highly conserved in mammalian and fungal $\Delta^{5,6}$-desaturases	30
-[b,c,d]	2 bp deletion leading to a frameshift and truncated protein (stop codon at position 194)	30
H269N[b]	recovered in an azole-resistant isolate with an *erg3* wild-type phenotype	30
P272L[b,c,d]	associated with H318N; residue highly conserved in mammalian and fungal $\Delta^{5,6}$-desaturases	30
H318N[b,c,d]	associated with P272L; located in a histidine-rich domain	30
Q327K[b,c,d]	residue conserved in mammalian and fungal $\Delta^{5,6}$-desaturases	30

The four histidine-rich conserved domains thought to constitute the active site of the enzyme are located at amino acids 226–230 (HX3H), 239–243 (HX2HH), 254–257 (HX2H) and 314–318 (HX2HH), in *C. albicans* and *C. dubliniensis*.
[a]Relative to the start methionine.
[b]Substitution recovered from a laboratory mutant (not a clinical isolate).
[c]Mutation likely to be responsible for an impaired Erg3p activity [either recovered as a single substitution or in combination with substitution(s) not associated with azole resistance in an azole-resistant isolate].
[d]Involvement of this substitution (alone or in combination with others) in azole resistance has been confirmed by heterologous gene expression in *S. cerevisiae*.

virulence of the two *erg3* mutants, supporting the theory of the contribution of hyphal growth to virulence. Interestingly, as shown by other authors, the reduced virulence trait of *erg3* mutant isolates is not necessarily linked to reduced kidney fungal burden (not performed in our study).[7,28] However, we cannot exclude the possibility that a specific genetic background in these strains, not link to the Erg3p impairment, could be responsible for the attenuated virulence. Firm evidence would require functional complementation of the *ERG3* allele.

In conclusion, this study gives further convincing evidence that *C. albicans erg3* mutants can develop following fluconazole therapy. We demonstrated for the first time the critical role of substitutions at residue 193, as well as of a novel large deletion, both having a dramatic impact on Erg3p function and azole resistance. Such *erg3* mutants could have an important impact on antifungal management strategies, due to cross-resistance between azoles and polyene antifungal drugs. However, their lower *in vivo* virulence, which probably explains their rather low frequency, could question their real impact in the clinical setting. Finally, these results also demonstrate that not only *ERG11* but also other genes involved in the ergosterol biosynthesis pathway must be investigated when azole resistance is suspected.

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Transparency declarations

None to declare.

Supplementary data

Tables S1 and S2 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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